Possibility of novel hexose conjugation of a d-type trichothecene by trichodiene synthase gene-disrupted mutants of *Fusarium* species

Keywords

biosynthesis; d-type trichothecene; feeding; *Fusarium graminearum*; *Fusarium sporotrichioides*; glycoconjugation; t-type trichothecene

Trichothecenes are a large group of mycotoxins, produced by several fungi such as *Fusarium*, *Myrothecium* and *Spicellum*. Among them, trichothecene-producing *Fusarium* species are notorious in agriculture because they infect important crops including wheat and corn, and accumulate trichothecenes in grains. For example, *Fusarium sporotrichioides* produces T-2 toxin and HT-2 toxin, whereas *F. graminearum* produces deoxynivalenol (DON) and nivalenol (NIV).

Trichothecenes are proposed to be divided into two groups, t-type and d-type, based on biosynthetic pathways. T-type trichothecenes are produced through the second cyclization of isorichotriol mainly in *Fusarium* species, while d-type trichothecenes are produced via the second cyclization of isorichodiol in non-*Fusarium* species (Fig. 1A)\(^1\). T-type trichothecenes have a modified group at the C-3 position, whereas d-type trichothecenes do not. Once the second cyclization proceeds, no exchange between d-type and t-type trichothecenes occurs.

Since we have succeeded in producing a novel unnatural trichothecene by utilizing the biosynthetic pathways of two fusaria, *F. sporotrichioides* and *F. graminearum* (manuscript in revision), challenges of creation of novel trichothecenes have been addressed by utilizing different trichothecene-producing genera. In trichothecene-producing species, TRI5, trichodiene synthase, catalyzes the first cyclization of farnesyl pyrophosphate (Fig. 1A)\(^1\); thus, TRI5 gene-disrupted *Fusarium* (ΔTri5) loses the ability to produce trichothecenes, but still keeps the other trichothecene biosynthetic genes. To produce novel d-type trichothecenes by means of enzymes of *Fusarium* trichothecene producing, trichodermol (TDmol, a d-type trichothecene obtained from *Spicellum roseum*), was fed to a ΔTri5 mutant of *F. sporotrichioides* (NBRC 9955; T-2 toxin producer) (FsΔTri5, manuscript in revision) and a ΔTri5 mutant of *F. graminearum* (MAFF 111233; 4-acetylNivalenol [4-ANIV] producer) (FGDS)\(^3\). The products at 48 h were analyzed by TLC and LC/MS/MS. As a positive control, isorichodermol (ITDmol, a t-type trichothecene intermediate) was fed to the Tri5-deleted mutants. If the biosynthetic enzymes work properly, ITDmol should be metabolized to T-2 toxin in FsΔTri5\(^4,5\), and to 4,15-diacetylNivalenol (4,15-diANIV) and 4-ANIV in FGDS\(^6\).

The ethyl acetate extracts from the cell culture of the disruption mutants fed with TDmol were analyzed by TLC, and trichothecenes were visualized by the 4-(p-nitrobenzyl)pyridine-tetraethylenepentamine method\(^7\), but no spots indicative of trichothecenes including TDmol were detected in either mutant. The products of the disruption mutants fed with TDmol were analyzed by LC/MS/MS. In the standard TDmol, extracted ion chromatograms (XIC) of m/z 251.164 ± 0.025 and 268.191 ± 0.025, corresponding to [TDmol + H]\(^+\) (m/z 251.1642) and [TDmol + NH\(_4\)]\(^+\) (m/z 268.1907), yielded peaks at 3.65 min with the MS/MS spectrum of [TDmol + H]\(^+\) as shown in Fig. 1B. In agreement with TLC, neither TDmol nor other trichothecene peaks were detectable in the samples of the disruption mutants obtained at 48 h. One of possible explanations for disappearance of TDmol is that TDmol was conjugated with hexose such as glucose. Thus, XIC of m/z 413.217 ± 0.025 and 430.244 ± 0.025, corresponding to [TDmol-hexoside + H]\(^+\) (m/z 413.2170) and [TDmol-hexoside + NH\(_4\)]\(^+\) (m/z 430.2435) was searched for. As a result, we found an XIC peak at 2.99 min in both proton and ammonium ion adducts in the samples of these Tri5-deleted mutants, indicating increased hydrophilicity of the product with faster retention time (2.99 min) than that of the substrate (TDmol, 3.65 min). The corresponding MS/MS spectra of [TDmol-hexoside + NH\(_4\)]\(^+\) were exactly the same between those of the disruption mutants (Fig. 1C, 1D). These spectra are similar to that of TDmol under m/z 251.164, suggesting that TDmol was conjugated with hexose in these samples. Many possibilities have also been examined in XIC analysis including de-epoxidation, hydrolysis of epoxide, hydroxylation at C-15, acetylation at C-15, or conjugation of a hexose, di-hexose, tri-hexose, sulfate
or their combinations; however, no obvious XIC was obtained except for conjugation of hexose. In the positive control of the Tri5-deleted mutants fed with ITDmol and obtained at 48 h, T-2 toxin was found in the extracted sample of Fs∆Tri5 whereas 4,15-diANIV and 4-ANIV were found in the sample of FGD5, as expected. This means that the trichothecene biosynthetic enzymes except for TRI5 worked properly here.

In this study, ITDmol that has a hydroxy group at the C-3 position was metabolized as expected in both Tri5-deleted mutants. Trichothecene-producing fusaria have trichothecene 3-O-acetyltransferase (TRI101), supposedly a self-defense enzyme, because a trichothecene with an acetyl group at C-3 is much less toxic than the corresponding trichothecene with a hydroxy group at C-3. Once ITDmol is produced after the second cyclization in fusaria, it is immediately acetylated at C-3 to produce isotrichodermin, which is much less toxic than ITDmol. Afterwards, a series of trichothecene biosynthetic enzymes work to produce final products in each Fusarium species.

On the other hand, TDmol without a hydroxy group at the C-3 position could not be detoxified by TRI101. Thus, TDmol may not be accepted as a possible substrate in biosynthetic pathways of fusaria. Instead, fusaria apparently detoxified this TDmol by glycoconjugation to increase hydrophilicity. Given that TDmol has a hydroxy group only at the C-4 position, conjugation of a hexose was assumed to occur at C-4. Although conjugation of trichothecene with glucose at C-3 in plants has often been reported, a trichothecene conjugated with a hexose at C-4 has never been reported so far. This phenomenon awaits further elucidation to understand the protective mechanisms of fusaria against trichothecenes.
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References


